THE EFFECT OF PIPETTING ON THE CONCENTRATION OF HOMOGENEOUS SPORE SUSPENSIONS

PART II

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A concentrating effect produced by the pipetting of suspensions of spores of *B. subtilis* has been followed by using a spectrophotometer to determine optical densities of suspensions of spores and volumetric dilutions of such suspensions made by pipetting. The behaviour of suspensions of spores in capillary tubes has been investigated and used to explain in part the concentrating phenomenon.

METHODS of assessing the viable count of microorganisms involve at some stage the procedure of volumetric dilution. Gerrard (1959) has shown that serious errors can be introduced during the pipetting of spore suspensions of *P. spinulosum*, in preparation for roll tube counts. In a communication to the British Pharmaceutical Conference 1960, Gerrard and Porter reported that suspensions of spores of *P. spinulosum* became more concentrated on pipetting unless precautions were observed. This concentrating effect was studied by determining optical densities of the suspensions. The use of the spectrophotometer for this work was based on the observation that a linear relation existed between the gravimetric dilution factor and optical density for a given spore suspension at the wavelength employed. The phenomenon was attributed to the fact that in the pipetting of a spore suspension, a relatively spore-free film of liquid is retained on the pipette wall. Thus, during a successive series of pipettings, as in a serial dilution, the total volume of vehicle retained on the walls of the pipettes can cause a marked concentrating effect.

The importance of this effect in bacteriological work, led to the present investigation of the behaviour of spores of *Bacillus subtilis* during pipetting procedures.

There is a considerable dimensional difference between the fungal and bacterial spores; *P. spinulosum* spores are spherical in shape and have a diameter about 5μ, whereas, *B. subtilis* spores are ellipsoidal to cylindrical and measure approximately 1·5 μ by 0·8 μ.

EXPERIMENTAL AND RESULTS

Preparation of Spore Suspensions

10 day cultures of *B. subtilis* N.C.T.C. 3610 were prepared on nutrient Agar slopes (Oxoid Granules CM. 3) contained in 250 ml. flasks incubated at 37°. The spores were harvested in sterile water and washed three times. The washing process consisted of depositing the spores from aqueous suspension by centrifuging (R.C.F. 3,100 for 15 min.) and resuspending the packed cells in a fresh volume of sterile water. Suspensions prepared in this way were stored in the refrigerator to allow vegetative

forms to sporulate. Microscopic examination of stained preparations of samples from the suspensions indicated that the suspensions consisted of spores only.

Pipettes

Cleaning of pipettes. It was found that unless particular care was taken with the cleaning of pipettes the concentrating effect described was not apparent. The use of Teepol to aid the cleansing process had to be discontinued since, even after copious rinsings, sufficient of the surface-active agent remained on the walls of the pipettes to interfere with the practical work.

The method adopted was to rinse the pipettes thoroughly in chromic acid, and allow them to soak overnight in this acid. After repeated

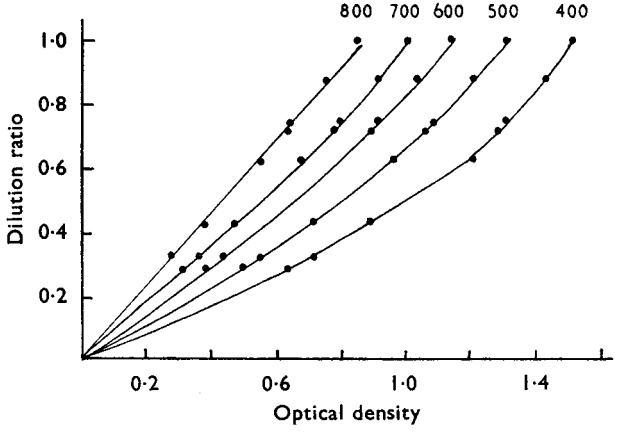

FIG. 1. The relation between dilution ratio and optical density. The figures at the top of the curves are wavelengths in mμ.

rinsing with distilled water in an automatic rinser, the pipettes were rinsed in absolute ethanol then ether and oven dried. The pipettes were finally sterilised in a hot-air-oven at 150° for 1 hr.

Choice and accuracy of pipettes. For this work which is essentially of a preliminary nature, Grade B pipettes designed to deliver their entire contents were used. These pipettes were graduated to 0·01 ml. and had an error of ± 0·02 ml. Pipettes of capacity 1 to 2 ml. were used.

Spectrophotometric Techniques

The Instrument. A Unicam S.P. 500 was used for the work described in this paper. All glass, optically matched 1 cm. cells were used with a Tungsten lamp as the light source.

The relationship between gravimetric dilutions of suspensions of B. subtilis *spores and optical density. (i) Choice of a suitable wavelength.* A heavy suspension of washed spores of *B. subtilis* was prepared, as previously described. The optical density of this suspension was measured. A series of gravimetric dilutions of this suspension were made in distilled

water and the optical densities of the diluted suspensions measured. Wavelengths in the range of 400 to 1,000 mμ were used for these measurements.

The relationship between dilution ratio (weight of master suspension/ weight of master suspension + water) and optical density became linear only at wavelengths of 800 mμ and over (Fig. 1). The direction of the non-linearity shown by the curves for wavelengths below 700 mμ indicates that lower optical densities are obtained than would be expected, at the higher concentration values. This would indicate that at wavelengths below 700 mμ, the more concentrated suspensions show a pronounced secondary scatter of light. The secondary scatter of light has the effect

TABLE I

OPTICAL DENSITY OF SUSPENSIONS AT VARIOUS DILUTION LEVELS

Wavelength in mμ	800	700	600	500	400	Dilution ratio
Original	0·855	1·022	1·150	1·375	1·530	—
1	0·760	0·930	1·0375	1·216	1·448	0·878
2	0·650	0·805	0·920	1·094	1·322	0·752
3	0·640	0·790	0·900	1·065	1·300	0·721
4	0·5575	0·695	0·8075	0·975	1·2125	0·629
5	0·382	0·480	0·665	0·720	0·895	0·428
6	0·290	0·370	0·445	0·560	0·721	0·330
7	0·2525	0·322	0·392	0·505	0·645	0·296

Specific gravity of suspension at room temperature = 0·9999.
Correlation of optical density at 800 mμ and dilution ratio — r = 0·998.

of redirecting the light, already scattered once out of the transmitted beam, so that some of it is returned to the original beam.

(ii) *Demonstration of linear relationship.* Having chosen a suitable wavelength range, a number of master suspensions of spores were prepared and the relationship between optical density (measure at 800 mμ) and gravimetric dilution ratio was found for each (Table I). In every case a positive linear relationship was found to exist between optical density of a suspension and its gravimetric dilution ratio. A statistical treatment of these results showed that a high degree of correlation was present between the two factors ($r = 0{\cdot}998$).

The relation between volumetric dilutions of spore suspensions of B. subtilis *made by pipette and the optical densities of such diluted suspensions. Experiment* (1). Five suspensions of washed spores of *B. subtilis* were prepared and their optical densities at 800 mμ determined. These suspensions will be referred to as master suspensions A, B, C, D and E (Table II).

Volumetric dilutions of the master suspensions were made by pipetting samples of suspension directly into a spectrophotometer cell and adding water from a burette (Class B 10 ml., tolerance ± 0·04 ml.).

The contents of the cell were thoroughly mixed by shaking and the optical density of the contained diluted suspension measured at 800 mμ.

The values obtained for replicate dilutions of the master suspensions are given in Table II. In every instance it will be seen that a concentrating effect is apparent in that the observed optical density of a diluted spore suspension is always in excess of the calculated value for that particular dilution.

Experiment (2). Two master suspensions of washed spores were prepared (F and G) and volumetric dilutions made with water. The procedure of making the dilutions differed from that used in Experiment (1)

TABLE II

VOLUMETRIC DILUTIONS OF SPORE SUSPENSIONS USING A 1 ML. PIPETTE
EXPERIMENT (1)

Suspension	Theoretical optical density	No. of replicate experiments	Observed optical density		*Observed density of suspension* × 100/ *expected density*
			Mean	Range	
Master A	—	—	0·88		
1 ml. (A) + 2·5 ml. H_2O ..	0·25	10	0·275	0·26–0·29	$\frac{0·275}{0·25} \times 100 = 110$
Master B	—	—	0·92		
1 ml. (B) + 2·5 ml. H_2O ..	0·26	6	0·285	0·28–0·29	$\frac{0·285}{0·26} \times 100 = 109$
Master C	—	—	1·20		
1 ml. (C) + 2·0 ml. H_2O ..	0·40	9	0·445	0·44–0·47	$\frac{0·445}{0·40} \times 100 = 110$
Master D	—		0·45		
1 ml. (D) + 1·25 ml. H_2O ..	0·20	3	0·245	0·24–0·25	$\frac{0·245}{0·200} \times 100 = 121$
1 ml. (D) + 1·50 ml. H_2O ..	0·18	3	0·215	0·21–0·22	$\frac{0·215}{0·18} \times 100 = 119$
1 ml. (D) + 1·0 ml. H_2O ..	0·23	3	0·265	0·26–0·27	$\frac{0·265}{0·23} \times 100 = 114$
Master E			0·88		
1 ml. (E) + 4 ml. H_2O ..	0·18	3	0·205	0·20–0·21	$\frac{0·205}{0·18} \times 100 = 114$
1 ml. (E) + 5 ml. H_2O ..	0·15	3	0·155	0·15–0·16	$\frac{0·155}{0·15} \times 100 = 103$
1 ml. (E) + 9 ml. H_2O ..	0·09	3	0·105	0·10–0·11	$\frac{0·105}{0·09} \times 100 = 116$

in that 2 ml. pipettes were used to measure the volumes of suspension and only the first ml. from each was delivered. As in Experiment (1) the dilutions were made directly into spectrophotometer cells and optical densities measured after thorough agitation of the contents of each cell. The values obtained for replicate dilutions of the master suspensions are given in Table III. It will be seen that no concentrating effect of the master suspension was obtained.

Study of Flow of Spores in a Capillary Tube

A capillary tube of elliptical cross section was attached vertically with plasticine to the stage of a microscope the optical axis of which was horizontal, the capillary tube having its major axis of cross section normal to the optical axis.

The spore suspension was introduced into the capillary tube through a polythene cannula and a needle and syringe barrel as shown in Fig. 2,A. The syringe acted as a reservoir the height of which could be varied by a

laboratory jack, and hence the rate of flow and position of meniscus in the capillary could be controlled.

When a descending column of suspension was observed through the microscope, stream line flow was seen, with peripheral spores moving more slowly and central spores more rapidly, than the meniscus.

TABLE III

VOLUMETRIC DILUTIONS OF SPORE SUSPENSIONS USING A 2 ML. PIPETTE
EXPERIMENT (2)

Suspension	Theoretical Optical density	No. of replicate experiments	Observed optical density		Observed density of suspension × 100/ expected density
			Mean	Range	
Master F			0·80		
1 ml. + 3 ml. H_2O	0·20	6	0·195	0·19–0·20	$\frac{0·195}{0·200} \times 100 = 97·5$
Master G			1·12		
1 ml. + 3 ml. H_2O	0·28	6	0·268	0·26–0·28	$\frac{0.268}{0·280} \times 100 = 95·7$

Slower moving peripheral spores, on approach of the meniscus, were projected into the faster central stream and not apparently left behind on the capillary wall above the meniscus (Fig. 2,B).

Observation of suspension flow in a 5 mm. bore tube showed that in accordance with the Poiseuille equation, the meniscus moved at half the rate of a spore in the centre of a tube.

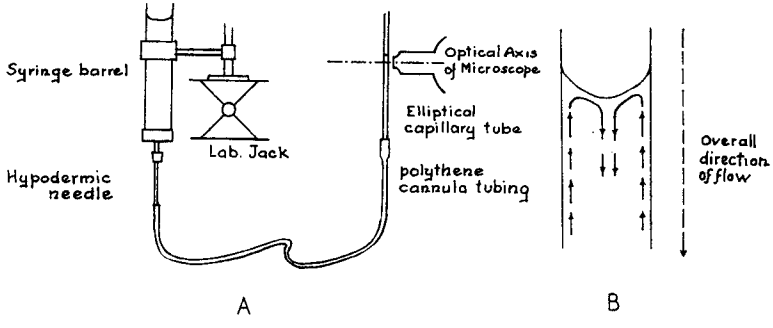

FIG. 2. A. Microscopical observation of behaviour of a *B. subtilis* spore suspension flowing down a capillary tube.
B. Relative movement of peripheral spores compared with that of meniscus.

Hence, if a delivery of 1 ml. is made from a full 2 ml. pipette, few or none of the spores originally at the periphery of the upper half of the 2 ml. of suspension will be ejected, and no concentrating effect should be apparent in the 1 ml. delivered.

DISCUSSION

It has long been recognised that the dilution procedures, involving the pipetting of suspensions of microorganisms, that form a part of any technique for determining viable count, can be a source of error. Ingram

and Eddy (1953) warned of the potential errors attendant upon the pipetting of suspensions of vegetative bacterial cells during dilution procedures, in preparation for viable count determinations. They found that the bacterial cells adhered to the walls of a pipette in such numbers that their dislodgement during the use of the same pipette for several successive dilutions could introduce serious errors. Errors introduced in this way may contribute to discrepancies in the final viable count of a given suspension, as reported by Fisher, Thornton and Mackenzie (1922). (See also Synder, 1947.)

To reduce this potential source of error, Bullock, Keepe and Rawlins (1949) have calibrated pipettes to deliver a determined volume of liquid between two calibration marks. Such pipettes will, indeed, give reproducible results in counts carried out with identical apparatus on a comparative basis. If, however, conditions for a series of dilution procedures are not identical, and this includes the precise position of the calibration marks on the stem of the pipettes, errors will become apparent.

When suspensions of spores of *B. subtilis* are placed in capillary tubes and examined with the microscope, the spores are seen to assume a definite uniform motion as the liquid column moves. A "funnelling movement" is seen to occur with the result that a fast flowing column of spores is formed in the centre of the capillary, leaving a relatively spore-free film of liquid on the wall of the capillary. This, in effect, means that at each pipetting the retention of spore-free liquid in the pipette has a concentrating effect on the spore suspension.

It has been shown that with pipettes which are designed to deliver their entire contents (i.e. not the volume between two fixed marks), the concentrating effect as judged by optical density readings can be about 7 per cent (see Table II). If, however, only a portion of the contents of such pipettes are delivered e.g. 1 ml. from a 2 ml. pipette, the concentrating effect can be negligible (see Table III).

This would suggest that one possible method of overcoming the concentrating effect, would be to calibrate pipettes to deliver a given volume of liquid between two points, the position of the points in relation to the entire length of the liquid column being a critical factor. The design and use of such pipettes for dealing with suspensions of spores of *P. spinulosum* has been described by Gerrard (1959).

As has been pointed out, the cleaning of glassware is a critical factor, in experiments of the type described. It was found that even minute traces of grease were sufficient to make the concentrating effect a variable factor. Surface-active cleaning agents, such as Teepol, left a fine film on the glassware which, also, detracted from the constancy of the concentrating phenomenon.

Earlier work described by Gerrard (1959), in which he followed the concentrating effect attendant upon pipetting of suspensions of spores of *Penicillium spinulosum*, showed the effect to be more marked and reproducible than the effect described here with bacterial spores. The reason for this might well lie in the comparative sizes of the two spores (viz. diameter about 3–5 μ for *P. spinulosum* and 1·5–2 μ for *B. subtilis*). It

has been found that a pipette of the type used in these experiments holding a liquid column of 16 cm. and having an internal diameter of 3 mm., will retain 30 mg. of liquid. This would correspond to a film of liquid along the interior of the pipette of 16 cm. length and 2 μ thick. A film of thickness of a similar diameter to a bacterial spore, might well be expected to retain these more readily than the much larger mould spores.

It is evident that many variable factors are at work and that detailed investigations will be necessary before the concentrating effects described can be followed on a truly quantitative basis.

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